

The DNA-Binding Properties of Two Heat Shock Factors, HSF1 and HSF3, Are Induced in the Avian Erythroblast Cell Line HD6

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Avian cells express three heat shock transcription factor (HSF) genes corresponding to a novel factor, HSF3, and homologs of mouse and human HSF1 and HSF2. Analysis of the biochemical and cell biological properties of these HSFs reveals that HSF3 has properties in common with both HSF1 and HSF2 and yet has features which are distinct from both. HSF3 is constitutively expressed in the erythroblast cell line HD6, the lymphoblast cell line MSB, and embryo fibroblasts, and yet its DNA-binding activity is induced only upon exposure of HD6 cells to heat shock. Acquisition of HSF3 DNA-binding activity in HD6 cells is accompanied by oligomerization from a non-DNA-binding dimer to a DNA-binding trimer, whereas the effect of heat shock on HSF1 is oligomerization of an inert monomer to a DNA-binding trimer. Induction of HSF3 DNA-binding activity is delayed compared with that of HSF1. As occurs for HSF1, heat shock leads to the translocation of HSF3 to the nucleus. HSF3 exhibits the properties of a transcriptional activator, as judged from the stimulatory activity of transiently overexpressed HSF3 measured by using a heat shock element-containing reporter construct and as independently assayed by the activity of a chimeric GAL4-HSF3 protein on a GAL4 reporter construct. These results reveal that HSF3 is negatively regulated in avian cells and acquires DNA-binding activity in certain cells upon heat shock.

Heat shock genes are transcriptionally induced upon exposure of cells to physiological conditions, including stresses such as heat shock, heavy metals, oxidative stress, and amino acid analogs, and during nonstressful conditions such as cell growth, differentiation, and viral infections (27). The effects of heat shock and other stresses on heat shock gene transcription are mediated through interactions of the heat shock element (HSE) that is composed of at least three pentanucleotide modules (nGAAn) arranged as contiguous inverted repeats (33) with heat shock factor (HSF), the inducible transcriptional activator that regulates the transcription of these stress-inducible genes (25). In the budding yeasts *Saccharomyces cerevisiae* and *Kluyveromyces lactis*, HSF binds to HSE constitutively as a trimer and stimulates transcription upon heat shock (22, 49, 50). During deactivation of the heat shock response, the C-terminal activator domain is unmasked and becomes phosphorylated (9, 21, 32). In contrast, HSF does not bind to the HSE under normal growth conditions in the fission yeast *Schizosaccharomyces pombe*, *Drosophila melanogaster*, and higher eukaryotes but acquires HSE-binding activity by oligomerization to a trimer upon exposure to stress conditions (17, 29, 50, 57, 58, 61, 62). HSF trimers exhibit cooperative interactions between adjacent and distantly spaced HSEs as measured by DNA binding studies and transcriptional activation (3, 63).

Recently, HSF genes from a number of eukaryotes have been cloned, thus allowing a comparative analysis to identify regulatory domains. The HSF family includes HSF1, HSF2, and HSF3 in higher eukaryotes (31, 35, 39, 42). Among the higher eukaryotes, three domains are conserved; these domains correspond to the DNA-binding domain (15, 20, 56, 59), an amino-terminal leucine zipper which is essential for oli-

gomerization (34, 51), and a carboxyl-terminal leucine zipper which is not present in the HSF of budding yeasts (12, 16, 31, 35, 39, 40, 42, 54). The carboxyl-terminal leucine zipper appears to have a critical role in maintaining HSF in a non-DNA-binding state, presumably by interfering with the leucine zippers which are required for trimer formation (31, 36, 64). The carboxyl-terminal 100 amino acids of HSF1 contains the transcriptional activation domain, whose activity is negatively regulated by sequences within the central region of HSF1 (18, 44).

One of the major questions about heat shock gene regulation is the identity of the sensor for physiological stress. Several lines of evidence suggest that the DNA-binding activity of HSF is regulated by the environment of the cell and may not be intrinsic. First, alteration of the temperature at which cells are growing modulates the response to a given temperature (2). Second, human HSF1 transfected into *Drosophila* cells or into tobacco protoplasts is activated at a lower temperature than is required in human cells (11, 54). One candidate as a component of the stress-sensing apparatus is the molecular chaperone hsp70 (13). The amount of free hsp70 correlates with the induction of HSF and the heat shock response (5, 6). Consistent with this observation, overexpression of hsp70 reduces the heat shock response and accelerates the attenuation of activated HSF (28). Moreover, a complex of activated HSF and hsp70 can be detected during attenuation of the heat shock gene transcription (1, 5). As the stable association of HSF and hsp70 without stress could be detected but not regulated by heat shock (37), hsp70 is thought to affect its regulatory activity through transient interactions with HSF (26).

All three avian HSFs are expressed in most cells and tissues (31), and the DNA-binding activity of each of the three HSFs is negatively regulated in the cell. HSF1 responds to the classical inducers of the heat shock response, whereas HSF2 is induced during hemin-induced differentiation of erythroleukemia cells and is constitutively active during early mouse development (4, 30, 38, 47). Comparison of the DNA-binding prop-

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erties of the three factors revealed that neither HSF3 nor HSF1 expressed in vitro had DNA-binding properties, whereas HSF2 exhibited constitutive DNA-binding activity. Upon heat shock or treatment with a nonionic detergent, only HSF1 acquired DNA-binding activity, indicating that HSF1 and HSF3 were not identical in their regulatory properties. These observations suggest unique roles of HSF3 (31). In this report, we examine the biochemical and cell biological properties of HSF3 and demonstrate that HSF3 and HSF1 DNA-binding activities are coactivated by heat shock in avian erythroblast HD6 cells and that these events are accompanied by the translocation of HSF3 into nucleus and conversion of HSF3 from a dimer to a trimer form.

MATERIALS AND METHODS

Plasmid constructions. The full-length chicken HSF1 and HSF3 cDNAs were modified by PCR mutagenesis to contain *EcoRI* sites (41) and ligated in frame in the pGEX-2T vector to create pGEX2T-HSF1 and pGEX2T-HSF3, respectively (48). The corresponding construct containing the chicken HSF2 cDNA flanked by *BamHI* sites was used to create pGEX2T-HSF2, which lacks the carboxyl-terminal 45 amino acids as a result of the location of the *BamHI* site. For overexpression of chicken HSF1, HSF2, and HSF3 in cultured cells, the full-length cDNAs were placed under the control of the human β -actin promoter (19). The *EcoRI* fragments of pCHSF1 (1.8 kb) and pCHSF2 (2.3 kb) and the *EcoRI*-*HpaI* fragment of pCHSF3-14 (1.5 kb) were blunt ended with DNA polymerase I Klenow fragment, ligated with the *BamHI* linker for HSF1 or *SalI* linker for HSF2 and HSF3, and inserted into the pH β APr-1-neo expression vector, creating pH β -HSF1, pH β -HSF2, and pH β -HSF3.

The chimeric GAL4-HSF constructs (pGAL4-HSF1 and pGAL4-HSF3) were generated by PCR to amplify fragments encoding residues 209 to 491 of HSF1 and residues 20 to 467 of HSF3, using oligonucleotide primers cHSF1-4 (5'-GCGAATTCCTTAGGACACGGTGGGGTCCTT-3') plus cHSF1-22 (5'-GCGAATTCCTTAGGACACGGTGGGGTCCTT-3') and cHSF3-8 (5'-GCGAATTCCTTAGGACACGGTGGGGTCCTT-3') plus cHSF3-31 (5'-GCGAATTCCTTAGGACACGGTGGGGTCCTT-3'), respectively. Following digestion with *EcoRI*, the PCR products were subcloned into pCMX-GAL4, which contains the amino-terminal 147 amino acids corresponding to the GAL4 DNA-binding domain (55).

Expression of chicken HSF1, HSF2, and HSF3 in *Escherichia coli* and preparation of lysates. Cultures of *E. coli* DH1 transformed with pGEX2T-HSF1, pGEX2T-HSF2, or pGEX2T-HSF3 were treated with 0.4 mM isopropylthiogalactopyranoside (IPTG) for 3 h. The cells were pelleted and resuspended in 50 mM Tris-Cl (pH 8.0)-2 mM EDTA-10 μ g of lysozyme per ml for 20 min at room temperature. After three cycles of freeze-thawing and sonication, the lysates were centrifuged at $10,000 \times g$ for 20 min at 4°C. The pellets were resuspended with 50 mM Tris-Cl (pH 8.0)-2 mM EDTA-1% Nonidet P-40-100 mM NaCl and centrifuged as described above. The pellets were resuspended with 50 mM Tris-Cl (pH 8.0)-2 mM EDTA-1% Nonidet P-40-100 mM NaCl and incubated for 1 h at 4°C. After centrifugation, the pellets were resuspended with the same buffer containing 1 M urea without Nonidet P-40. The insoluble pellets were solubilized in 20 ml of buffer containing 30 mM Tris-Cl (pH 8.0), 30 mM NaCl, 8 M urea, and 1 mM dithiothreitol (DTT). These solutions were subsequently dialyzed against the same buffer containing 4 M urea (1 liter) for 12 h at 4°C and then against the same buffer without urea (6 liters). After centrifugation, the solutions were stored at -20°C.

Generation of antisera against chicken HSF1, HSF2, and HSF3. The bacterial lysates were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and the respective fusion proteins were excised, electroeluted, and used to immunize rabbits. The anti-HSF3 serum (α HSF3) contained low levels of cross-reactive antibodies to HSF2, which were removed by chromatography on immobilized glutathione *S*-transferase (GST)-HSF2. Three specific antisera, termed α HSF1 β , α HSF2 α , and α HSF3 γ , were obtained. For supershift experiments in Fig. 10, we generated antisera for a carboxyl-terminal region of each HSF.

Cell culture and preparation of whole cell extracts, cytoplasmic extracts, and nuclear extracts. Quail fibroblasts (QT6 cells) were maintained in a medium containing Dulbecco modified Eagle medium (DMEM) supplemented with 5% fetal calf serum, 2% chicken serum, and 2% tryptose phosphate broth. Chicken embryo fibroblasts (CEF cells) were maintained as described previously (31). For the transfection studies, CEF cells were grown in DMEM containing 5% fetal calf serum, 2% chicken serum, and 2% tryptose phosphate broth. The chicken erythroblast cell line HD6 (7) was provided by J. D. Engel (Northwestern University, Evanston, Ill.) and maintained in HD6 medium (434 ml of DMEM, 40 ml of fetal calf serum, 10 ml of chicken serum, 5 ml of 100 \times nonessential amino acids, 10 ml of 0.5- μ g/ml hypoxanthine in 0.5 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.3], 2.5 ml of dimethyl sulfoxide, 0.5 ml of 0.1-mg/ml biotin) (10).

Whole cell extracts were prepared as previously described (29), and subcellular fractionation was performed as described by Dignam et al. (14).

Western blot (immunoblot) analysis. Proteins were analyzed by SDS-PAGE and transferred to nitrocellulose filters by electrophoretic transfer with a buffer containing 5 mM sodium tetraborate. The filters were blocked with 5% dry milk in phosphate-buffered saline (PBS) for 1 h at room temperature and incubated with a 1:500 dilution of rabbit antiserum against each HSF or anti-hsp90 serum (a gift from I. Yahara, Tokyo Metropolitan Institute for Medical Research) in PBS with 2% dry milk. After being washed with PBS, the filters were incubated with a 1:1,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Cappel, Durham, N.C.) for 30 min at room temperature, and then signals were detected by the Amersham ECL system.

Immunofluorescence study. CEF cells were cultured on glass coverslips for 16 h, fixed with 50% methanol-50% acetone at -20°C for 20 min, and then washed with PBS. The same results could be obtained when cells were fixed with either absolute methanol or 4% paraformaldehyde. After blocking with 10% normal goat serum in PBS, cells were incubated with 1:300-diluted α HSF3 in 10% normal goat serum in PBS and then with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G antibody (1:200 dilution) (Cappel). The coverslips were washed and mounted in 80% glycerol in 10 mM Tris-HCl (pH 7.5) on a coverglass. Immunolabeled cells were visualized by fluorescence microscopy (Nikon, Tokyo).

Gel filtration. Whole cell extracts (500 μ l containing 500 to 1,000 μ g of protein) of each cell type were applied on a Superdex 200 HR column with a fast protein liquid chromatography apparatus (Pharmacia). The samples were eluted at 0.3 ml/min with a buffer containing 1% glycerol, 20 mM Tris-HCl (pH 7.9), 200 mM KCl, and 1.5 mM MgCl₂. The fractions (0.5 ml) were precipitated with trichloroacetic acid (10%, final concentration), washed with acetone, dried, suspended in gel sample buffer, and analyzed by SDS-PAGE and Western blotting. The peak positions of HSFs were estimated by quantifying signals of each fraction. K_{av} values were determined for each peak by using the formula $K_{av} = (V_e - V_o)/(V_i - V_o)$. V_e is the elution volume of the protein, V_o is the column void volume (6.9 ml), and V_i is the column volume (24 ml). The Stokes radius (R) was determined from the protein standard curve of K_{av} versus $\log(R)$. The protein standards used were thyroglobulin (669 kDa, 85.0 Å [1 Å = 0.1 nm]), ferritin (440 kDa, 61.0 Å), aldolase (158 kDa, 48.1 Å), and albumin (67 kDa, 35.5 Å).

Sedimentation analysis. Whole cell extracts (80 μ l containing 250 to 500 μ g of protein) were diluted with 120 μ l of 20 mM HEPES (pH 7.9) (final glycerol concentration of 10%) and loaded on top of 10 to 40% glycerol gradients containing 20 mM HEPES (pH 7.9), 100 mM NaCl, 5 mM MgCl₂, 0.5 mM EDTA, and 1 mM DTT. The gradients were centrifuged in an RPS65T rotor (Hitachi, Ibaragi, Japan) at 40,000 rpm for 40 h at 4°C and divided into 250- μ l fractions. Each fraction was precipitated with trichloroacetic acid and analyzed by SDS-PAGE and Western blotting. The peak volumes were determined by quantifying HSFs or standard proteins in each fraction. S values ($s_{20,w}$) of HSFs were determined from the protein standard curve of S value versus peak volume. The protein standards used were aldolase (158 kDa, 7.35S), bovine serum albumin (67 kDa, 4.3S), and chymotrypsinogen A (25 kDa, 2.55S). The frictional ratio was calculated as previously described (45, 46, 58).

Gel mobility shift assay. Gel mobility shift assay was performed as previously described (29). The antibody supershift experiments were performed with 0.5 to 2.0 μ l of 1:10-diluted antiserum in PBS and 1.0 to 2.0 μ l of cell lysates in a total volume of 10 μ l. After incubation on ice for 20 min, an oligonucleotide probe mixture containing ³²P-labeled self-complementary ideal HSE oligonucleotides (39) was added, and the mixture was incubated on ice for 20 min and analyzed on 4% native polyacrylamide gels.

DNase I footprint analysis. Radiolabeled probes were prepared (23) from a linearized plasmid pKD3 by digestion with *SalI* (coding) or *HindIII* (noncoding), end labeling, and excision of the fragment containing the human hsp70 promoter from bp -4 to -188 with *HindIII* or *SalI*, respectively. The labeled fragments were purified from a polyacrylamide gel.

The bacterial lysate containing GST-HSF3 (8.0 μ g) was mixed with thrombin (40 ng) and CaCl₂ (final concentration, 2 mM) to a final volume of 20 μ l and incubated at 25°C for 1 h. Labeled probe (1 μ l), 0.5 μ l of poly(dI-dC) · poly(dI-dC) (5 mg/ml), 62.5 μ l of 2 \times binding buffer (20 mM Tris-Cl [pH 7.5], 100 mM NaCl, 2 mM EDTA, 10% glycerol), and 36 μ l of water were used for the binding mixture. After 20 μ l of the binding mixture and 5 μ l of the lysate (0 to 2.0 μ g) were mixed and incubated on ice for 30 min, 25 μ l of ice-cold buffer consisting of 5 mM CaCl₂ and 10 mM MgCl₂ was added, and then the mixture was treated with DNase I as previously described (23).

Transfection, CAT assay, and luciferase assay. Transient overexpression of HSFs in QT6 or CEF cells was obtained by using calcium phosphate transfection (3a). The precipitates containing calcium phosphate and DNA were added to the cells at 1 h after a change to fresh medium, incubated for 12 h, and then washed three times with PBS. The chloramphenicol acetyltransferase (CAT) assay was performed with extracts of cells transfected with pH β -HSF plasmids, 5 μ g of LSNWT as a reporter gene (60), and 0.5 μ g of a plasmid containing the human β -actin promoter upstream of the luciferase gene as an internal control. Cell lysates were prepared by the standard method with 0.25 M Tris-Cl as the lysis buffer, and the CAT and luciferase activities were assayed.

For the luciferase assay, COS7 cells were maintained in DMEM with 10% fetal calf serum and transfected by using calcium phosphate containing 20 μ g of the

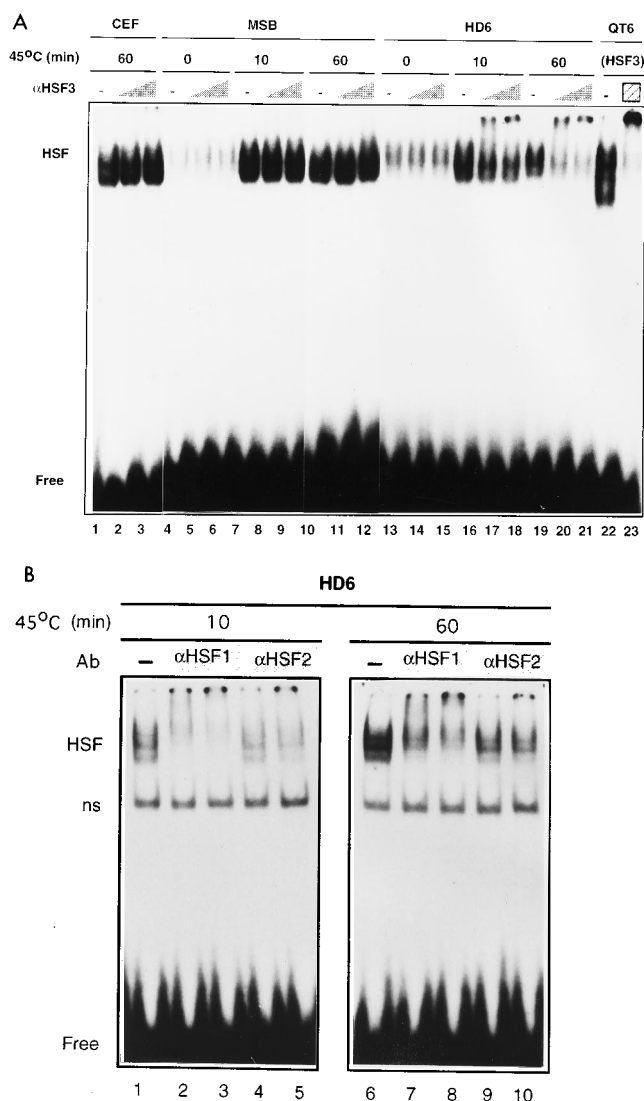


FIG. 4. HSF3 DNA-binding activity is activated in vivo upon heat shock. (A) Analysis of the DNA-binding activity of HSF3 in CEF, MSB, and HD6 cells. Cells were heat shocked at 45°C for 10 and 60 min, and then whole cell extracts were prepared for gel shift assay. After incubation of cell lysates (10 μ g) and α HSF3 γ (0.5 or 1.0 μ l of 1:10-diluted serum to a final volume of 10 μ l in PBS) on ice for 20 min, 32 P-labeled probe was added. These mixtures were loaded on 4% native polyacrylamide gel. Lanes 22 and 23 correspond to lanes 12 and 13 in Fig. 3. (B) DNA-binding activities of HSF1 and HSF2 in HD6 cell lysates after heat shock at 45°C for 10 min (left) and 60 min (right). Preimmune serum (lanes 1 and 6) (1.0 μ l of 1:10-diluted serum), α HSF1 β (lanes 2, 3, 7, and 8), and α HSF2 α (lanes 4, 5, 9, and 10) (0.5 or 1.0 μ l of 1:10-diluted serum) were used as described above. ns represents the nonspecific binding activity, which varies according to the lot of poly(dI-dC) \cdot poly(dI-dC) (data not shown). Ab, antibody.

extracts from heat-shocked CEF or MSB cells (Fig. 4A, lanes 1 to 12), whereas the HSF complex induced by heat shock in HD6 cells was supershifted by α HSF3 (Fig. 4B, lanes 16 to 21). From these observations, we conclude that HSF3 DNA-binding activity can be activated by heat shock; however, regulation of this activity occurs in a cell-type-specific manner. Since HSF1 is also expressed in HD6 cells, we examined whether both DNA-binding activities were induced. Extracts from heat-shocked HD6 cells were incubated separately with α HSF1 or α HSF2 (Fig. 4B). As indicated in Fig. 4B, α HSF1 entirely supershifted the HSE-binding activity at 10 min of heat shock

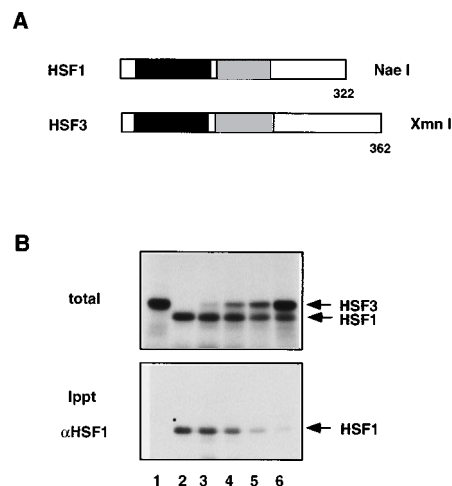


FIG. 5. HSF3 and HSF1 do not form heterotrimers. (A) Schematic representation of the truncated products of HSF1 (322 amino acids) and HSF3 (362 amino acids) which lack leucine zipper 4. (B) The cDNAs for HSF1 and HSF3 were linearized with *Nae*I and *Xmn*I, respectively. The truncated in vitro transcripts were cotranslated in reticulocyte lysate at different ratios in the presence of [35 S]methionine. Three microliters of the in vitro translation mixture was analyzed by SDS-PAGE (top). The remaining aliquot of the reaction mixture was analyzed by immunoprecipitation (Ippt) with α HSF1 (bottom). The position of each product is indicated.

(lanes 2 and 3), with less of an effect at the 60-min heat shock time point (lanes 7 and 8), whereas α HSF2 had little or no effect on the DNA-binding activity (lanes 4, 5, 9, and 10). These results reveal that HD6 cells activate the DNA-binding activities of both HSF1 and HSF3 in response to heat shock.

One of the potential questions posed by coactivation of HSF1 and HSF3 is whether the DNA-binding complexes detected by gel shift analysis contain heterotrimers of HSF1 and HSF3. To examine this question, we cotranslated HSF1 and HSF3 DNA transcripts in which the carboxyl-terminal zipper motif was deleted as indicated in Fig. 5A, thus resulting in expression of the constitutively active form of each factor (data not shown and reference 30). The in vitro translation product of HSF1 contains 322 amino acids, whereas HSF3 contains 362 amino acids and is readily distinguished by SDS-PAGE (Fig. 5B, top). In vitro translation reaction mixtures containing both HSFs were subjected to immunoprecipitation with α HSF1. As shown in Fig. 5B (bottom), HSF3 is not immunoprecipitated by α HSF1, which suggests that HSF1 and HSF3 do not form stable heterotrimers.

Heat shock induces the translocation of HSF3 to the nuclei of heat-shocked cells. The subcellular distribution of HSF3 was examined in control and heat-shocked cells by using biochemical fractionation followed by Western blot analysis and indirect immunofluorescence. Fractionation of control and heat-shocked cells into cytoplasmic and nuclear fractions reveals that HSF3 in HD6 cells resides entirely in the cytoplasm of control cells (Fig. 6A, first row) and translocates to the nucleus within 10 min of heat shock. Following a return to nonshock conditions (37°C for 2 h of recovery), HSF3 becomes cytoplasmic. The rapid heat shock-dependent reversible translocation described for HSF3 also occurs simultaneously for HSF1 in HD6 cells (Fig. 6A, second row). In contrast to the stress-dependent translocation observed for HSF3 and HSF1, HSF2 is relatively unaffected by heat shock and remains distributed in the cytoplasm and nucleus (Fig. 6A, third row) (43, 46).

The subcellular distribution of HSF3 was also examined in CEF cells by using biochemical fractionation (Fig. 6B) and

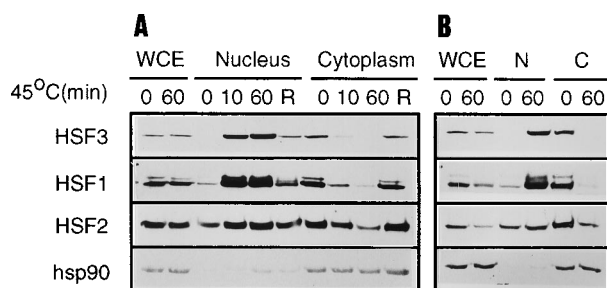


FIG. 6. Subcellular localization of HSF1 and HSF3 in control and heat-shocked HD6 and CEF cells. HD6 (A) and CEF (B) were heat shocked for the indicated periods or allowed to recover (lanes R) at 37°C for 2 h after heat shock at 45°C for 1 h. Whole cell extracts (WCE), nuclear extracts (N), and cytoplasmic extracts (C) were prepared as described in Materials and Methods. Each extract (40 μ g) was analyzed by SDS-PAGE, transferred to a nitrocellulose filter, and incubated with the respective antiserum. HSF3, HSF1, HSF2, and, as a control, cytosolic heat shock protein hsp90 were blotted.

indirect immunofluorescence (Fig. 7). Control CEF cells exhibit diffuse cytoplasmic staining, and upon heat shock, HSF3 was primarily detected as punctate nuclear granules (Fig. 7). The results obtained by indirect immunofluorescence are consistent with the data obtained upon biochemical fractionation of heat-shocked CEF cells, which reveals that HSF3 translocates into the nucleus upon heat shock (Fig. 6B).

The oligomeric state of HSF3 is affected by heat shock. To further characterize the biochemical properties of HSF3, we analyzed the oligomeric state of HSF3 in extracts from control and heat-shocked HD6 and CEF cells by using gel filtration and sedimentation analysis. The results of gel filtration on whole cell extracts from control and heat-shocked CEF or HD6 cells revealed that HSF3 in control cells has a molecular mass of 390 kDa, which is between the molecular sizes of control and heat-shocked HSF1 (Fig. 8A and E, fractions 19). Upon heat shock, the molecular mass of HSF3 increases to 670 kDa in both cell types (Fig. 8B and F, fractions 16 and 17). The oligomeric state of HSF1 was also examined in CEF and HD6 cells. The molecular mass of control HSF1 is approximately 160 kDa (Fig. 8C and G, fractions 22 and 23), and following heat shock, HSF1 has a molecular mass of approximately 670 kDa (Fig. 8D and H, fractions 15 to 17) (46, 58). A minor population of HSF1 with a molecular mass of approximately 360 kDa is detected in both control and heat-shocked lysates of CEF cells (Fig. 8C and D, fractions 19).

Additional evidence that HSF3 undergoes oligomerization was obtained by glycerol gradient sedimentation of extracts from control and heat-shocked CEF and HD6 cells and analysis of the fractions by Western blot analysis with α HSF1 and α HSF3 (Fig. 9). The S values were determined for control HSF3 (5.0S), heat-shocked HSF3 (7.9S), control HSF1 (3.0S), and heat-shocked HSF1 (7.5S). These values were consistent with the dimer-to-trimer transition of HSF3. From these two analyses, we determined the frictional ratios (f/f_0) of HSF3 and HSF1 in CEF cells by assuming that HSFs would not be modified and HSF3 would be a homodimer. The frictional ratios of HSF1 in control and heat-shocked cells were 1.81 and 2.04, respectively, and those of HSF3 in control and heat-shocked cells were 1.92 and 1.99, respectively. These results indicate that HSF3 is ellipsoid before and after heat shock.

The Stokes radii were determined for control HSF3 (64 Å), heat-shocked HSF3 (85 Å), control HSF1 (45 Å), and heat-shocked HSF1 (85 Å). These data are also consistent with the interpretation that HSF3 is a dimer in control cells and in a

trimeric state upon heat shock and that HSF1 is a monomer in control cells and a trimer upon heat shock.

The data on subcellular localization and oligomeric state of HSF3 in both HD6 and CEF cells reveal that heat shock leads to a translocation to the nucleus and oligomerization from an inert dimer to a trimer. However, HSF3 DNA-binding activity is detected only in HD6 cells.

HSF3 exhibits delayed DNA-binding activation kinetics. Our previous observations (Fig. 4) on the activation of HSF3 DNA-binding activity suggested the possibility that HSF1 and HSF3 were not coordinately activated. To further examine the kinetics of activation of the HSFs in response to heat shock, we prepared extracts from cells exposed to heat shock at 45°C for periods of up to 120 min (Fig. 10A) or to arsenite for periods of up to 6 h (Fig. 10B). The heat shock-induced HSE DNA-binding activity was rapidly induced and attained maximal levels within 5 to 10 min (Fig. 10A, PI panel lane 3). The relative levels of HSF1 and HSF3 DNA-binding activities were determined by performing antibody supershift experiments. HSF1 was rapidly induced within 2 min (α HSF1 panel), whereas HSF3 DNA-binding activity (α HSF3 panel) was detected between 20 and 40 min. As expected, the addition of α HSF2 did not affect the DNA-binding activity (α HSF2 panel) compared with the levels of HSE-binding activity in the absence of α HSF.

Additional support for the delayed kinetics of HSF3 activation was obtained by analysis of extracts of cells treated with sodium arsenite. The difference in activation kinetics of HSF1 and HSF3 is more dramatic with this condition of chemical stress (Fig. 10B). The DNA-binding activity detected between 0.5 to 1.5 h was supershifted only by addition of α HSF1, whereas α HSF3 generated a supershift complex after 2 to 3 h of arsenite treatment. The delay in activation of HSF3 DNA-binding activity measured by gel shift and supershift analysis

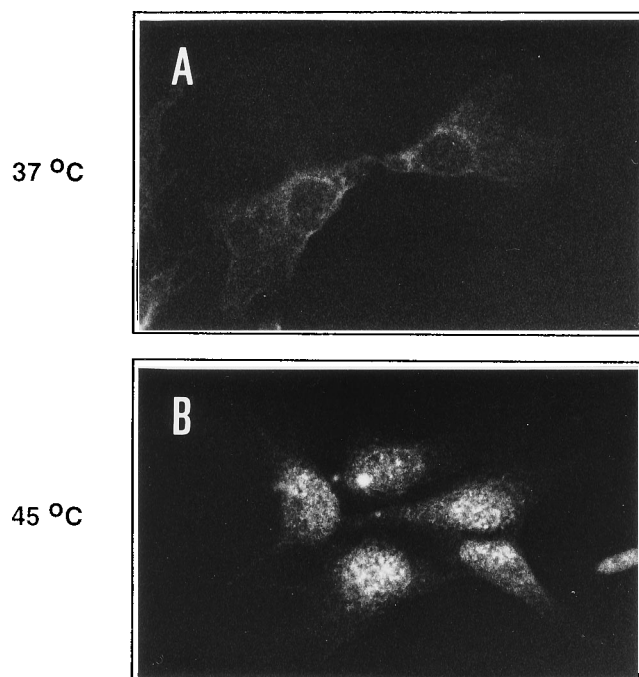


FIG. 7. Translocation of HSF3 to the nucleus in heat-shocked CEF cells. Unshocked (A) and heat-shocked (45°C for 60 min; B) CEF cells were subjected to indirect immunofluorescence analysis using α HSF3 and fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G antibody for the second antibody. Immunolabeled cells were photographed by fluorescence microscopy.

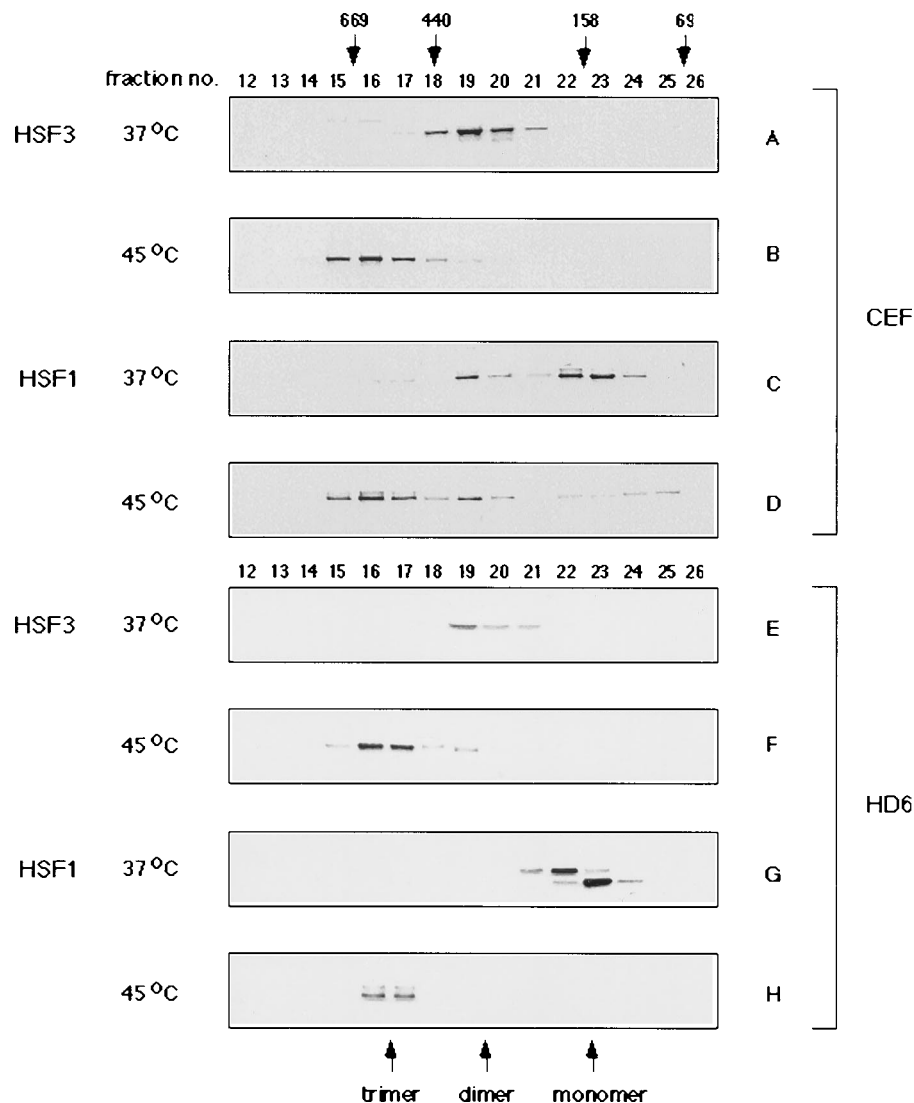


FIG. 8. Oligomeric state of HSF3 and HSF1 in control and heat-shocked CEF and HD6 cells determined by gel filtration. Whole cell extracts of CEF (A to D) and HD6 cells (E to H) with or without heat shock were prepared. These lysates were fractionated by gel filtration with a Superdex 200 HR column (Pharmacia). Proteins of each fraction were precipitated with trichloroacetic acid and analyzed by SDS-PAGE and Western blotting with specific antisera. The predicted elution positions of monomer, dimer, and trimer forms of HSFs are indicated at the bottom. The approximate elution positions of protein standards are indicated on the top (669 kDa, thyroglobulin; 440 kDa, ferritin; 158 kDa, aldolase; 69 kDa, bovine serum albumin).

was also observed as a delay in the kinetics of cytosol-to-nucleus translocation of HSF3 compared with HSF1 in arsenite-treated cells. HD6 cells were treated with arsenite and fractionated into cytosolic and nuclear extracts, and the levels of HSF1 and HSF3 were estimated by Western blot analysis (Fig. 11). Whereas HSF1 was rapidly translocated to the nucleus, the fraction of nucleus-localized HSF3 was delayed relative to HSF1, a result which is consistent with the delayed kinetics of HSF3 DNA-binding activity.

DNA-binding and transcription activation properties of HSF3. To further characterize the transcription activation properties of HSF3, we used *in vitro* footprint analysis of the recombinant protein, using the human *hsp70* promoter as a substrate. The human *hsp70* promoter has been extensively studied for DNA-protein interactions by using *in vivo* and *in vitro* footprint methodologies to demonstrate that HSF1 and HSF2 exhibit related footprints with characteristic differences in the occupancy of the HSE (23, 47). Using DNase I foot-

printing, we found that recombinant HSF3 protects a region of DNA corresponding to nucleotides -120 to -85 (coding strand) and -115 to -88 (noncoding strand) (Fig. 12A). The boundary of HSF3 interaction extends over all five pentamer-binding sites (monomer unit is nGAAn), which corresponds to the same boundaries previously observed for HSF1 interaction on the heat shock element of the *hsp70* gene (Fig. 12B) (23).

While these studies clearly establish that HSF3 exhibits many of the DNA-binding properties of HSF1, we have not established whether HSF3 has the activity of an activator or repressor. Addressing this issue directly is difficult because HSF1 and HSF3 are coexpressed and coactivated by heat shock. Consequently, the background of HSF1 activity would interfere with our ability to directly examine the *in vivo* transcription activation properties of HSF3. As an alternative approach, we examined the activity of a reporter gene in cells constitutively expressing HSF3. As shown in Fig. 13A, cotransfection of the HSF1 expression vector together with a reporter

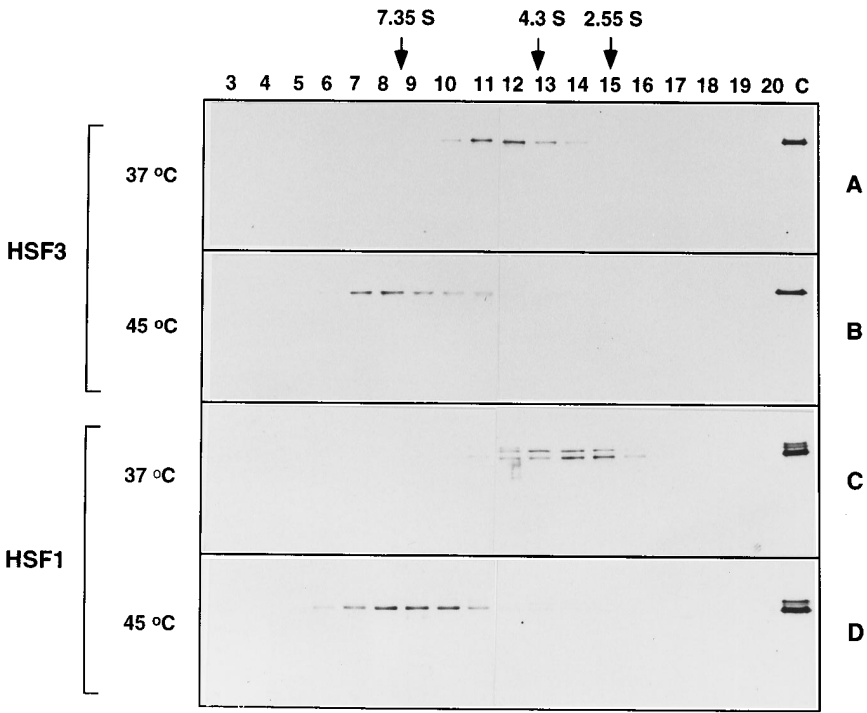


FIG. 9. Glycerol density gradient analysis of sedimentation properties of HSF3 and HSF1. Whole cell extracts were fractionated by glycerol gradient centrifugation (10 to 40%). Fractions were precipitated and analyzed by Western blot analysis. The whole cell extracts of CEF (20 μ g) were loaded as controls (lane C). The same profiles were detected in HD6 cell lysates (data not shown). The sedimentation positions of protein standards are indicated (7.35S, aldolase; 4.3S, bovine serum albumin; 2.55S, chymotrypsinogen A).

containing the minimal heat shock-responsive human hsp70 promoter upstream of the CAT gene (LSNWT) (60) resulted in a slight (1.4-fold) increase in CAT activity relative to cells without an expression vector. Cells cotransfected with the

HSF3 expression vector exhibited 15.7-fold-higher CAT activity relative to cells without an expression vector (Fig. 13A), indicating that overexpression of HSF3, which results in constitutive HSF3 DNA-binding activity, leads to transactivation

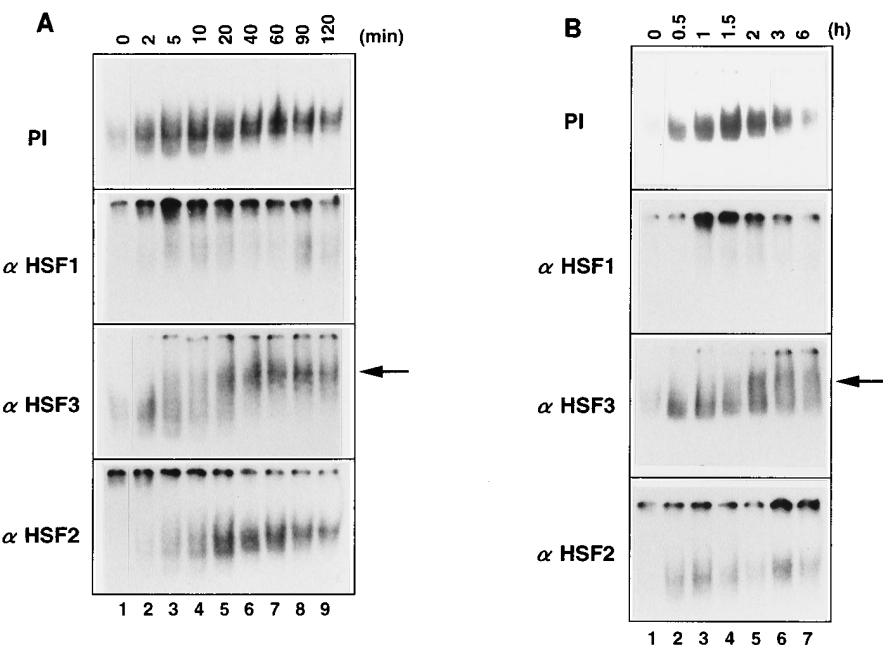


FIG. 10. Kinetics of DNA-binding activities of HSF3 and HSF1 during a time course of exposure to heat shock or arsenite treatment. (A) HD6 cells were heat shocked at 45 °C for the indicated times, and whole cell extracts were prepared. Cell lysates (10 μ l) were incubated with preimmune serum (PI) or an antiserum specific for a carboxyl-terminal region of each HSF (2 μ l of 1:10-diluted serum to a final volume of 10 μ l in PBS) on ice for 20 min; 32 P-labeled probe was added. The mixtures were loaded on 4% native gel. The arrow indicates the position of supershift bands detected after addition of α HSF3. (B) HD6 cells were treated with arsenite, and DNA-binding activity was analyzed as described above.

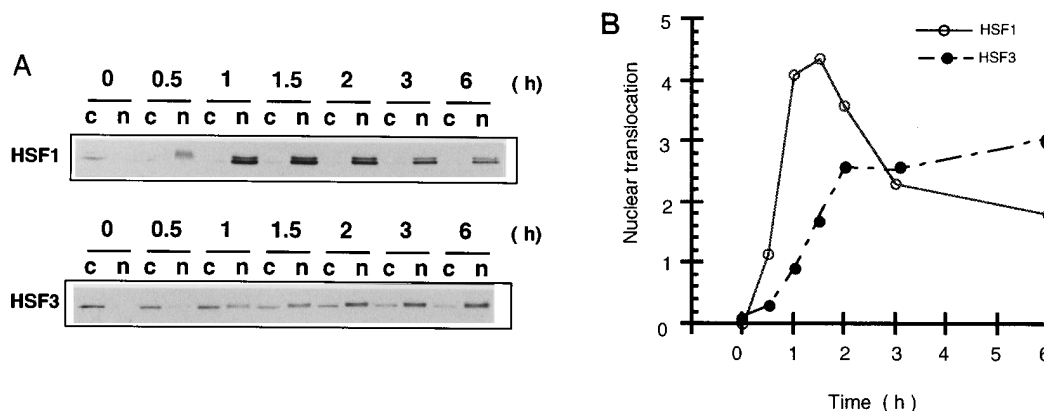


FIG. 11. Kinetics of the nuclear translocation of HSF3 and HSF1 after treatment with arsenite. HD6 cells were treated with sodium arsenite (100 μ M) for the indicated times, and cytoplasmic (c) and nuclear (n) fractions were prepared. Thirty micrograms of each lysate was subjected to SDS-PAGE, transferred to nitrocellulose, blotted with α HSF1 serum or α HSF3, and visualized on X-ray film by using the ECL system (A). The film was scanned, and intensity of the bands in the nuclear fraction was estimated by using NIH image analysis (B).

of an HSE-dependent reporter construct. We obtained independent evidence that HSF3 was a positive activator by transient transfection of a chimeric GAL4-HSF3 (amino acids 202 to 467) protein. The activity of GAL4-HSF3 was measured by cotransfection with a reporter plasmid containing the GAL4-binding site, and the activity was compared with that of a positive control, GAL4-HSF1 (amino acids 209 to 491) (55). Cells cotransfected with a GAL4-HSF1 expression vector and a GAL4-HSF3 expression vector exhibited 22.8- and 73.0-fold-higher luciferase activities relative to cells transfected with a GAL4 expression vector alone (Fig. 13B). Taken together, these data offer strong evidence that HSF3 has the properties of a transcription activator.

DISCUSSION

HSF3 was originally identified as a new member of the vertebrate HSF gene family on the basis of sequence relatedness with HSF1 and HSF2 (31). The three distinct members of the HSF family share approximately 40% sequence identity. However, the organization of each HSF, in particular the relative location and amino acid sequence of the DNA-binding domain and the leucine zippers, is highly conserved. HSF3 is ubiquitously expressed, and the DNA-binding properties are negatively regulated, presumably through intramolecular interactions involving the carboxyl-terminal zipper 4, as has also been suggested for HSF1 (31, 36). The analysis of the properties of HSF3 presented in this study reveals that it is an inert dimer and undergoes oligomerization to an active trimer form. However, in contrast with HSF1, acquisition of HSF3 DNA-binding activity in response to heat shock appears to be a cell-specific event.

While these studies have identified an *in vivo* cellular stress condition that leads to HSF3 activation, our current analysis is too limited to allow us to conclude whether the induction in HD6 cells reflects a novel form of regulation. We are puzzled by the observation that HSF3 DNA binding is detected only in HD6 cells, as HSF3 expression is not limited to specific avian tissues and cell lines. Activation of HSF3 DNA-binding activity is not restricted to the HD6 cell line, as we have found that the chicken B-lymphoblast cell line DT40 also induces HSF3 upon heat shock (53). Comparison of the effects of heat shock on HSF3 in embryo fibroblasts and HD6 cells reveals that HSF3 activity can be regulated at multiple distinct steps. In embryo fibroblasts, heat shock influences the biochemical properties of

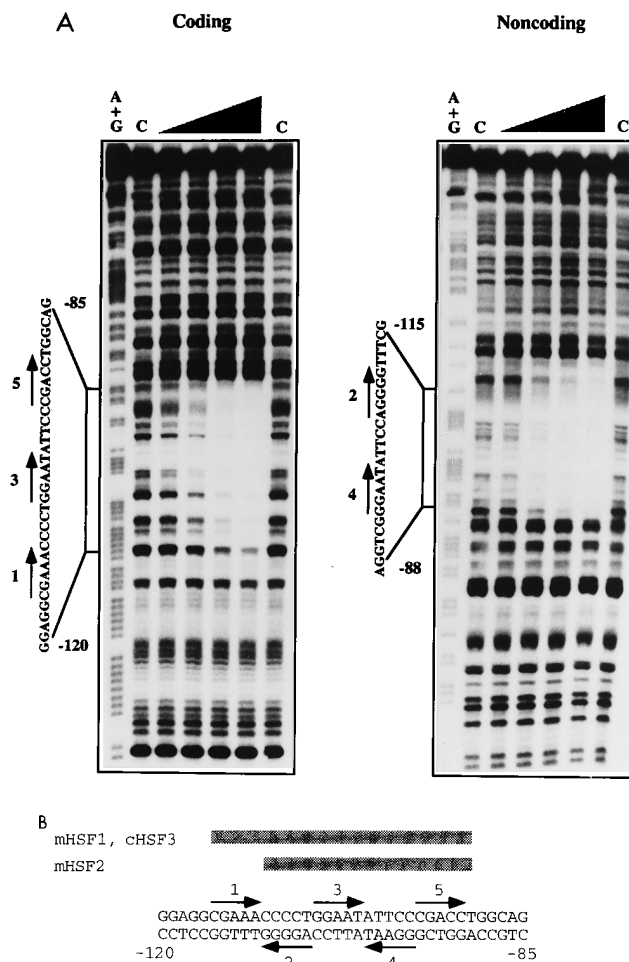


FIG. 12. *In vitro* DNase I footprint analysis of the hsp70 promoter, using recombinant HSF3. (A) DNase I footprinting analysis of HSF3 binding to the promoter on the human hsp70 gene. Coding and noncoding strand footprints are indicated, using increasing amounts of HSF3 (0.25, 0.5, 1.0, and 2.0 μ g) treated with thrombin to remove GST. Lanes: A+G, A and G ladders; C, DNase I digestion without HSF3. The sites of protection indicated on the left provide a minimum boundary for footprinting estimated from A+G reaction. The nGAAn motifs in the proximal HSE are indicated in the sequence. (B) Schematic illustration of the protection sites on the hsp70 promoter. The protection sites of mouse HSF1 and HSF2 (mHSF1 and mHSF2) (23) are compared with those of chicken HSF3 (cHSF3).

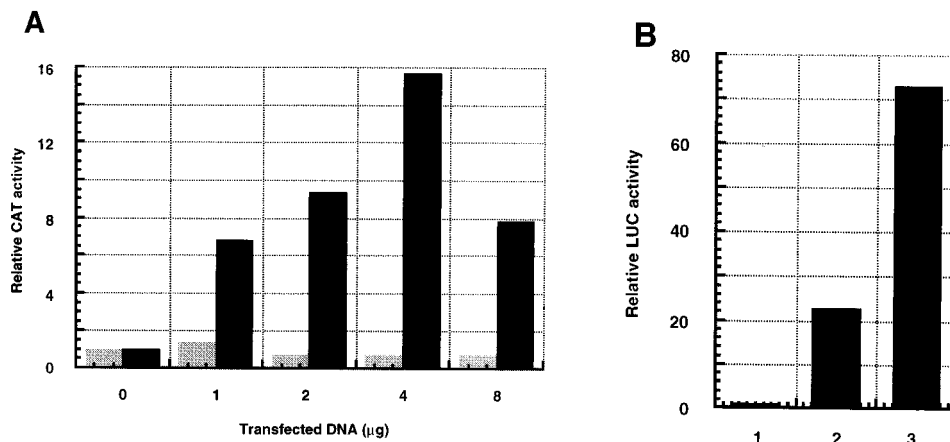


FIG. 13. HSF3 has the activity of a positive transcriptional activator. (A) Increasing levels of HSF3 expression vector pH β -HSF3 (black bar) or HSF1 expression vector pH β -HSF1 (gray bar) (amounts of DNA were adjusted to a total of 15 μ g with vector DNA) were cotransfected into CEF cells together with LSNWT (5 μ g) as a reporter and a construct containing human β -actin promoter upstream of a luciferase gene (0.5 μ g) as an internal control. At 12 h after transfection, cells were washed with PBS and incubated for another 24 h. The CAT activities of these cell lysates were analyzed by thin-layer chromatography. The amount of acetylated chloramphenicol was quantitated with a Molecular Dynamics PhosphorImager. (B) The chimeric GAL4-HSF1 and GAL4-HSF3 constructs corresponding to pCMX-GAL4 (bar 1), pGAL4-HSF1 (bar 2), and pGAL4-HSF3 (bar 3) were cotransfected into COS7 cells together with 5 μ g of reporter plasmid ptk-galp3-luc and 1 μ g of pSVCAT as an internal control. After 36 h of transfection, cell lysates were prepared and luciferase activities were assayed. The relative levels of luciferase activity of cells cotransfected with a GAL4 DNA-binding domain expression vector were normalized to the level of cells transfected with pCMX-GAL4, which was assigned a value of 1. The relative CAT activities used as internal controls were 13, 12, and 22, respectively.

HSF3 and results in nuclear translocation and oligomerization. Yet despite these events, HSF3 does not acquire DNA-binding properties in embryo fibroblasts as detected by the use of α HSF3 to detect DNA-protein complexes containing HSF3. In contrast, HSF3 expressed in HD6 cells undergoes translocation and oligomerization and exhibits DNA-binding activity. These data reveal that HSF3 DNA-binding properties can be regulated at a point following trimer formation and nuclear localization in a cell-specific manner. It is also possible that α HSF3 does not detect the HSF3 activated in embryo fibroblasts. Additional studies will be required to distinguish between these possibilities.

As to the role of HSF3 in the heat shock response of HD6 cells, our observations indicate that HSF3 has the properties of a positive activator as measured on HSE-containing reporter constructs. These data suggest that HSF3 could enhance the transcription of genes encoding heat shock proteins and molecular chaperones; however, the data presented here do not directly address this issue because of the presence of endogenous HSF1 activity. Therefore, the analysis of HSF3 as a transcriptional activator is based on an indirect approach in which the activity of a GAL4-HSF3 chimeric protein is assayed by using a GAL4 reporter construct from additional experiments in which the overexpression of HSF3 leads to the constitutive DNA-binding activity and transactivation of an HSE-containing reporter construct. Although it is difficult to directly compare the relative strengths of the transactivation domains of HSF3 and HSF1, our results suggest that HSF3 may contain a more potent activation domain.

The analysis of HSF3 also reveals a biochemical property previously described for HSF2, i.e., that HSF3 is an inert dimer which undergoes oligomerization to a trimer form (46). While the range of conditions that lead to activation of HSF2 has not been fully described, some examples include hemin-induced erythroid differentiation in human erythroleukemia cells, early murine embryogenesis, and murine spermatogenesis (30, 46). These conditions are distinct from the stress-related events that lead to activation of HSF1; consequently, HSF1 is induced by a broad range of conditions that lead to cell stress, and

HSF2 activity is unaffected by these conditions. Thus HSF1 and HSF2 exhibit complementary roles that extend the regulatory potential of the HSF family to respond to diverse conditions of physiological and environmental cell signalling and stress.

The coactivation of HSF1 and HSF3 in HD6 cells exposed to heat shock suggests the possibility of HSF regulatory redundancy, perhaps to ensure that the transcriptional activation of heat shock genes is both rapidly induced and sustained at a high level in response to extreme temperatures. In this context, it is worth noting that the optimal heat shock temperature for avian cells is 45°C, compared with 42°C for mammals. The possibility of a complementary role for these apparently redundant HSFs is supported by the rapid kinetics of HSF1 induction followed by HSF3 induction. This observation, together with our previous demonstration that the *in vitro* activation properties of HSF3 and HSF1 differ (31), indicates that these two factors are similar and yet have distinct regulatory features as measured by acquisition of DNA-binding activity. To establish the biological role for HSF3, however, it will be necessary to either inactivate or substantially reduce HSF1 expression, which would allow us to demonstrate whether HSF3 is itself sufficient to activate the expression of the endogenous heat shock genes.

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